

KIR3DL2/CpG ODN Interaction Mediates Sézary Syndrome Malignant T Cell Apoptosis

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We previously identified the NK cell receptor KIR3DL2 as a valuable diagnostic and prognostic marker for the detection of the tumoral T cell burden of Sézary syndrome (SS) patients. However, the function of this receptor on the malignant T lymphocyte population remained unexplored. We here demonstrate that engagement of KIR3DL2 by its recently identified ligand CpG oligodeoxynucleotide (ODN) induces the internalization of the receptor and leads to a caspase-dependent apoptosis of malignant T cells. This process of cellular death is correlated to a dephosphorylation of the transcription factor STAT3 (signal transducer and activator of transcription 3), which is found constitutively phosphorylated and activated in Sézary cells. Our results indicate that KIR3DL2 can directly promote SS malignant cell death through the use of CpG ODN.

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INTRODUCTION

Sézary syndrome (SS) is an aggressive leukemic and erythrodermic variant of cutaneous T cell lymphoma characterized by the presence of a clonal T lymphocyte population in the skin, lymph nodes, and peripheral blood. Numerous studies have tried to identify specific markers for an unequivocal detection of Sézary cells, which are currently identified on morphological criteria as oversized cells presenting an atypical cerebriform nucleus. However, this detection method is not entirely reliable because cerebriform mononuclear cells can be detected in the blood of healthy individuals and can also represent reactive or normal CD4⁺ T cells exhibiting a Sézary cell-like appearance (Duncan and Winkelmann, 1978; van der Loo *et al.*, 1981). Similarly, the antigen expression abnormalities reported so far, such as the loss of CD7, CD26, or CD3 expression (Edelman and Meyerson, 2000; Bernengo *et al.*, 2001; Rappl *et al.*, 2001), were not constantly observed for all patients, rendering the use of multiple markers more accurate for the detection of the circulating malignant cells (Klemke *et al.*, 2008). We previously reported that KIR3DL2 represents a specific cell surface marker for the evaluation of the circulating tumoral burden and for the follow-up of patients with SS (Bagot *et al.*, 2001; Poszepczynska-Guigne *et al.*, 2004; Ortonne *et al.*, 2006; Bouaziz *et al.*, 2010). KIR3DL2 is a member of the killer cell Ig-like receptor (KIR) family, whose ligand specificity was

assigned to HLA-A3 and -A11 through a peptide-specific interaction (Hansasuta *et al.*, 2004) and to HLA-B27 homodimer through a peptide-independent recognition (Kollnberger *et al.*, 2007). More recently, CpG oligodeoxynucleotides (ODNs) were also identified as KIR3DL2 ligands on NK cells, the interaction between CpG DNA and KIR3DL2 leading to the endocytosis of the ligand/receptor complexes and to NK cell activation (Sivori *et al.*, 2010).

The specific expression of KIR3DL2 in SS patient malignant cells prompted us to investigate its possible influence on mechanisms regulating tumoral cell growth and apoptosis. Cellular outcomes and signaling were analyzed following KIR3DL2 engagement by using its recently identified ligand CpG ODN.

RESULTS

KIR3DL2 internalization is induced in Sézary cells upon engagement with CpG ODN

Because CpG ODNs were shown to promote KIR3DL2 cell surface downmodulation on NK cells (Sivori *et al.*, 2010), we first tested whether this observation also applied to malignant Sézary cells. We observed that incubation of Sézary patients' peripheral blood mononuclear cells (PBMCs) with type A, B, or C CpG ODNs, but not with a control ODN, led to a significant downmodulation of KIR3DL2, corresponding to a 50% reduction in the receptor mean fluorescence intensity (MFI), at the surface of Sézary patients' CD4⁺ T cells (Supplementary Table S1 online). CpG ODN-C was preferentially used for the following experiments because it induced efficient cell surface modulation of KIR3DL2 and combined the immune effects of class-A and -B ODN on immune cells (Krieg, 2002; Vollmer *et al.*, 2004). To definitely assess that the expression level of KIR3DL2 was downmodulated on malignant cells, immunolabelings including an anti-TCRVβ mAb (that identifies the tumoral T cell clone) were performed on both the Sézary cell line Pno and PBMCs from Sézary

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Abbreviations: JAK, Janus kinase; MF, mycosis fungoides; MFI, mean fluorescence intensity; ODN, oligodeoxynucleotide; SS, Sézary syndrome

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patients whose TCRV β rearrangement of their malignant T cell clone was identified (Figure 1a). The resulting data demonstrated that ODN-C promoted KIR3DL2 downmodulation

on the cell line (Figure 1a, upper panel) and on the patients' malignant circulating T cell clone, as assessed by the decrease in receptor expression detected on the CD3⁺CD4⁺V β ⁺

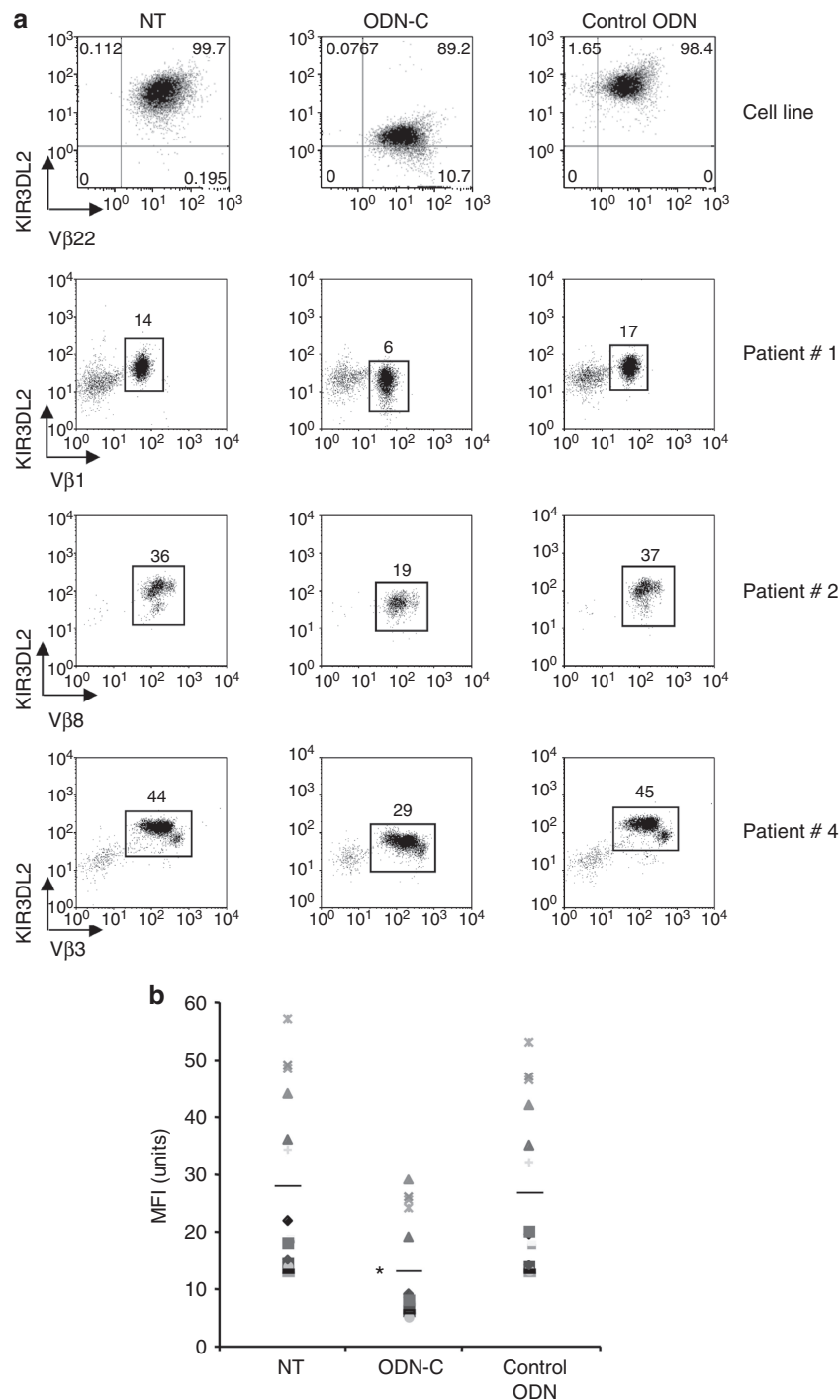


Figure 1. Downmodulation of KIR3DL2 expression on tumoral Sézary T-cell clone upon CpG ODN-C treatment. (a) The Sézary cell line (Pno) or PBMCs from Sézary patients were left untreated (NT) or incubated in the presence of CpG ODN-C or control ODN ($10 \mu\text{g ml}^{-1}$) for 8 or 48 hours, respectively. Cells were labeled with anti-KIR3DL2 mAb (Q66) plus FITC-conjugated goat anti-mouse IgM secondary antibodies, anti-TCRV β -PE, -CD3-PC5, and CD4-PC7 mAbs. TCRV β /KIR3DL2 staining corresponding to the gated CD3⁺CD4⁺ T lymphocyte population is shown. The mean fluorescence intensity (MFI) of KIR3DL2 labeling is indicated. (b) Graphical representation of KIR3DL2 MFI observed on the CD3⁺CD4⁺ T cells from Sézary patients ($n = 12$), following incubation in medium alone (NT) or supplemented with CpG ODN-C or control ODN. * $P = 0.004$ compared with NT condition. ODN, oligodeoxynucleotide; PBMCs, peripheral blood mononuclear cells.

T cell population (Figure 1a, patients one, two, and four). Such significant decrease in KIR3DL2 surface expression upon ODN-C treatment was obtained in samples from 12 different Sézary patients tested (Figure 1b; $P < 0.005$).

To directly visualize KIR3DL2 cellular location together with ODN-C, fluorescence microscopy analyses were performed. LAMP-1 was used as a marker for the endosomes/lysosomes compartments (Ebrahim and Thilo, 2011). In resting cells, KIR3DL2 was mainly detected at the plasma membrane (Figure 2a, upper panel). Similarly, FITC-conjugated ODN-C labeling of untreated cells led to the detection of a membrane-bound signal (Figure 2a, lower panel). After treatment with

control ODN, no major relocation of KIR3DL2 was detected (Figure 2b). In contrast, incubation of the cells with ODN-C led to a complete redistribution of KIR3DL2 that became detected in the LAMP-1 endo-lysosomal compartment (Figure 2c). The use of FITC-conjugated ODN-C allowed the detection of its co-internalization with KIR3DL2 (Figure 2d, upper panel) and its presence within the LAMP-1-positive intracellular compartment (Figure 2d, lower panel). Together, these data showed that binding of CpG ODN-C to KIR3DL2 promotes internalization of the receptor/ODN complexes in Sézary cells, similarly to what was reported in peripheral blood NK lymphocytes (Sivori *et al.*, 2010).

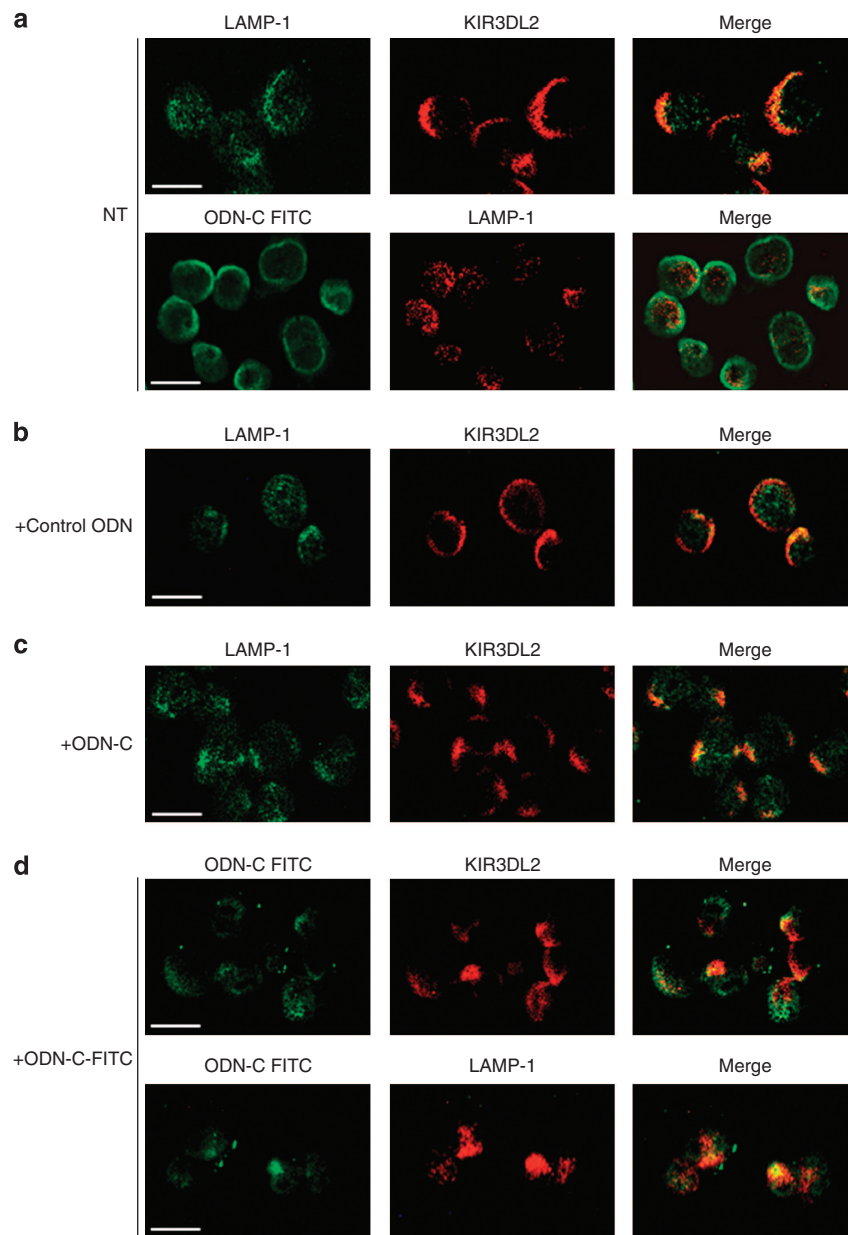


Figure 2. Internalization of KIR3DL2 following ODN-C treatment. Cells were left untreated (a) or incubated 6 hours in the presence of control ODN (b), ODN-C (c), or FITC-conjugated ODN-C (d). Immunolabelings were then performed using FITC-coupled ODN-C, anti-LAMP1, and/or anti-KIR3DL2 antibodies, as indicated. Scale bar = 10 μ m. ODN, oligodeoxynucleotide.

CpG ODN-C/KIR3DL2 interaction leads to Sézary cell apoptosis

To further investigate the consequences of KIR3DL2 triggering by CpG ODN-C in Sézary cells, we evaluated its role in the process of malignant T cell survival. Experiments performed on the Pno Sézary cell line highlighted a deleterious function for CpG ODN-C, with the detection of ~60% of apoptotic cells after 24 hours of treatment, whereas control ODN showed no effect on the cell line survival (Figure 3a). When similar experiments were performed on PBMCs from Sézary patients, no malignant cell death was detected in the presence of ODN-C at this time point (data not shown). Incubation time with CpG ODN-C or control ODN was therefore extended up to 12 days. Note that only patients showing a high tumoral burden and over 98% of KIR3DL2⁺ cells within their CD4⁺ T lymphocyte population were selected for these experiments, as exemplified with patient 15 (Supplementary Figure S1 online). Representative results obtained on PBMCs from patient 15 at days 3, 7, and 10 are shown in Figure 3b. Despite the detection of an increased spontaneous cell death during the time course, as assessed by both 7AAD labeling and cell count (see Figure 3b, NT panels and Supplementary Figure S2 online), addition of ODN-C resulted in a significant increase in the percentage of malignant (Vβ3⁺) cells that underwent apoptosis on days 7 and 10. In contrast, control ODN treatment did not modify the rate of cell death when compared with untreated cells. Furthermore, the survival of the non-tumoral cell populations (Vβ3⁻ cells) was not affected upon ODN-C treatment, with similar levels of Vβ3⁻ 7AAD⁺ cells being detected under all conditions of incubation and at all time points. Experiments performed on PBMCs isolated from healthy donors (*n*=4) revealed that normal CD4⁺ T cell viability was not affected by the presence of ODN-C (Supplementary Figure S3 online). Similar results were obtained on cells from eight patients (Figure 3c), with maximal ODN-C-induced apoptosis detected after 7–12 days of treatment depending on the patient.

CpG ODN-C treatment of Sézary cells results in caspase activation and phospho-STAT3 dephosphorylation

The induction of a caspase-dependent apoptotic pathway following incubation with ODN-C was investigated by immunoblotting on the Pno Sézary cell line and on CD4⁺ T cells isolated from patients (*n*=3) whose CD4⁺ T cell population was identified as entirely tumoral by performing TCRVβ/KIR3DL2 immunolabeling (Supplementary Figure S4 online). The obtained results showed that the levels of cleaved forms of caspase-7 and -3, and of their substrate PARP, were increased after incubation of both cell types with CpG ODN-C when compared with untreated cells (Figure 4). However, maximal activation of the caspase pathway was achieved after 24 hours of treatment for the Sézary cell line but required over 7 days for the patients' malignant cells, these delays of response being in agreement with the apoptosis data obtained by flow cytometry (see Figure 3). Together, these results showed that specific apoptosis of Sézary malignant cells is induced by CpG ODN-C treatment through a caspase-dependent pathway.

Previous studies have established that signal transducer and activator of transcription 3 (STAT3) can be constitutively

phosphorylated in Sézary cells and that treatments promoting its dephosphorylation lead to malignant cell death (Eriksen *et al.*, 2001; van Kester *et al.*, 2008; Zhang *et al.*, 2010). We therefore investigated the phosphorylation status of STAT3 in CpG ODN-C-treated cells. Experiments performed on the Pno cell line or purified CD4⁺ T cells of three patients (whose CD4⁺ T lymphocytes were all KIR3DL2⁺; Supplementary Figure S4 online) showed that a complete STAT3 dephosphorylation, which is not correlated to the degradation of the protein, occurred upon ODN-C treatment, whereas the phospho-STAT3 level was not modified in the presence of control ODN (Figure 5). Again, a much faster effect of ODN-C was seen in the Pno cell line when compared with Sézary patients' cells. Thus, the STAT3 dephosphorylation process was mainly completed after 8 hours of treatment in the Pno cell line, whereas it required 3 days for the patients' circulating malignant cells. Nevertheless, these data demonstrated that the CpG ODN-C-induced apoptosis of Sézary cells might be correlated to STAT3 dephosphorylation.

DISCUSSION

Advanced stages of SS are associated with aggressive tumors and poor prognosis. In this context, one critical issue was to identify a specific marker of Sézary cells allowing a reduction in diagnosis time and the unequivocal targeting of the circulating tumoral cells. We previously identified KIR3DL2 as a reliable marker of both skin-homing and peripheral blood malignant T cells (Bagot *et al.*, 2001; Poszepczynska-Guigne *et al.*, 2004). Because of its primary amino acid sequence, KIR3DL2 has an inhibitory role in NK and in cytotoxic CD8⁺ T cells. Moreover, KIR3DL2 expression has been reported in tumor-specific cytotoxic T cell clones isolated from T lymphocytes infiltrating human lung carcinoma, but its engagement did not result in an inhibition of cytotoxicity or cytokine release (Dorothee *et al.*, 2003). We now demonstrate that KIR3DL2 engagement by its recently identified ligand CpG ODN promotes the internalization of the receptor and the generation of apoptotic signals in Sézary cells.

For all patients' samples tested, we experienced on average a 50% reduction in KIR3DL2 MFI following CpG ODN treatment. This reduction did not increase with higher concentrations of CpG ODN (up to 25 μg ml⁻¹) or a longer exposure time (up to 12 days) (data not shown). In NK cells, KIR3DL2 internalization leads to the co-localization of CpG ODN-linked receptors with Toll-like receptor 9 (TLR9) in the endosomal compartment (Sivori *et al.*, 2010). It has therefore been suggested that, in these cells, KIR3DL2 may act as a carrier protein that brings CpG ODN to its receptor TLR9, resulting in NK cell activation. Despite the detection of TLR9 transcripts in Sézary cell lines and Sézary patient tumoral cells, we did not detect any TLR9 expression in these cells (data not shown). In addition, it has been reported that CpG and non-CpG ODN can co-stimulate mouse and human CD4⁺ T cells via a TLR9- and MyD88-independent mechanism (Landrigan *et al.*, 2011). Together, these data suggest the possibility of a KIR3DL2/CpG ODN-mediated effect with no TLR9 involvement in Sézary cells. This also points toward a distinct role for KIR3DL2 as a CpG ODN receptor in NK and

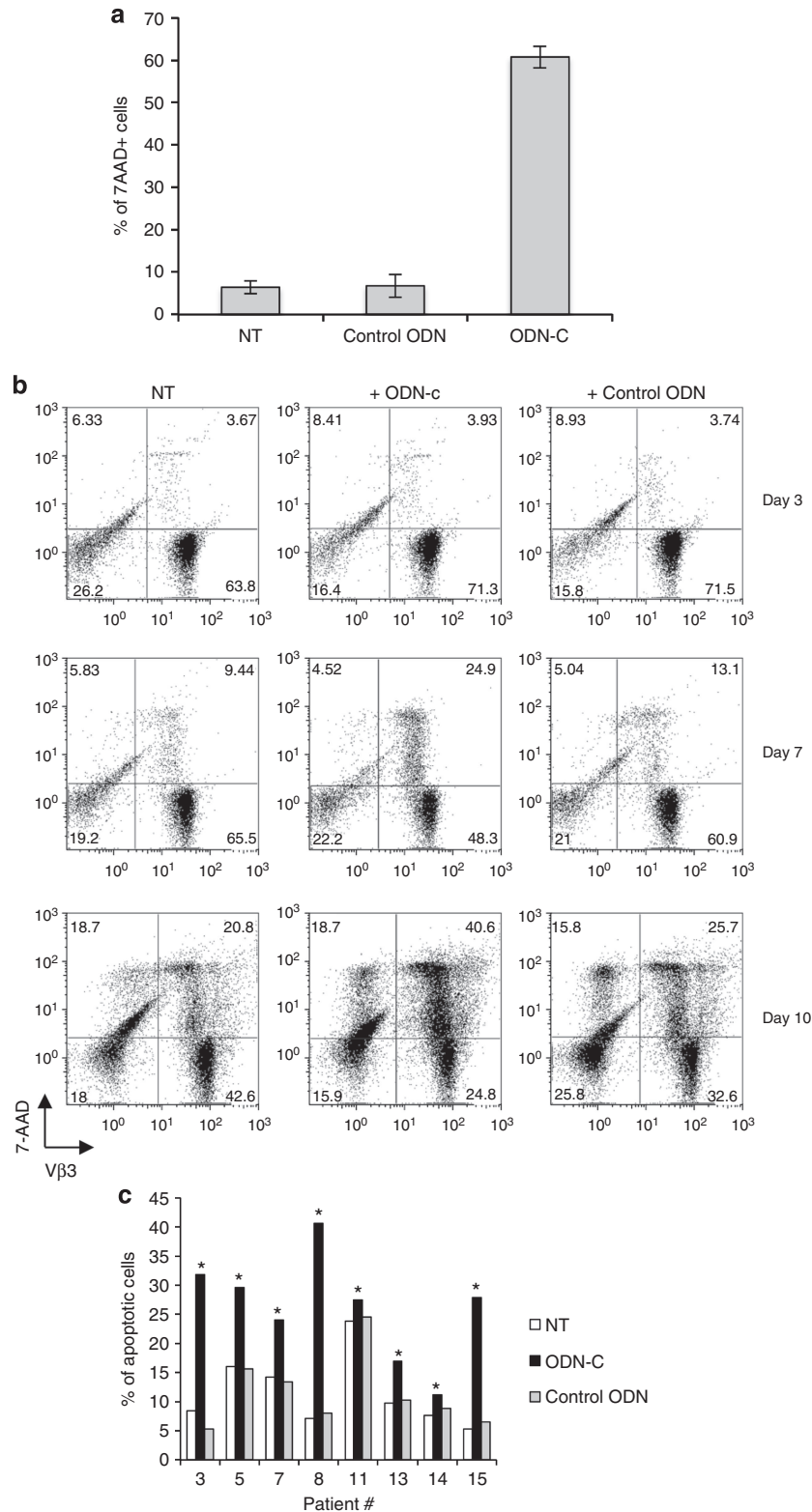


Figure 3. CpG ODN-C induces malignant Sézary cell apoptosis. (a) The Pno Sézary cell line was left untreated (NT) or incubated with CpG ODN-C or control ODN for 24 hours at 37 °C. Apoptotic cells were detected by 7AAD labeling. Mean \pm SD of four independent experiments is shown. (b) Sézary patient PBMCs were incubated with CpG ODN-C or control ODN at 37 °C. Immunolabeling was performed after 3, 7, or 10 days of incubation using anti-TCRVβ3-PE and -CD4-FITC mAb and 7AAD. (c) Graphical representation of the percentage of 7AAD-positive cells within the TCRVβ⁺CD4⁺ population of Sézary patients ($n=8$) after 7 days of incubation with CpG ODN-C (black) or control ODN (gray). * $P=0.006$. ODN, oligodeoxynucleotide; PBMCs, peripheral blood mononuclear cells.

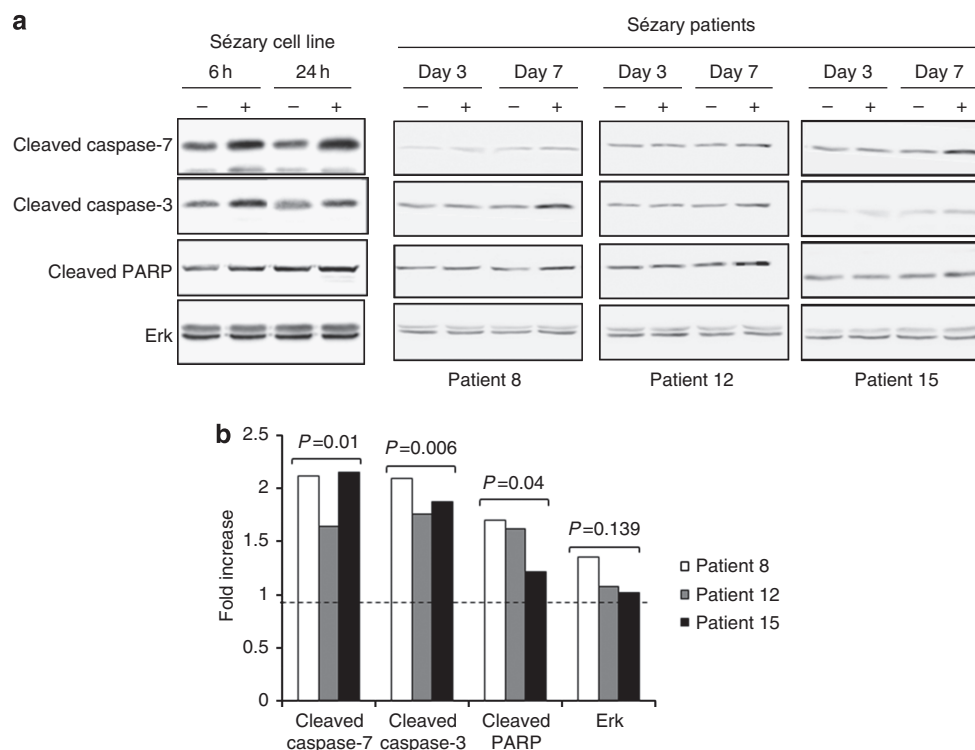


Figure 4. CpG ODN-C-induced apoptosis is associated with caspase activation. (a) The Sézary cell line or purified CD4⁺ T cells (98% of which were KIR3DL2⁺, see Supplementary Figure S4 online) from three Sézary patients were left untreated or incubated with CpG ODN-C. Post-nuclear lysates were prepared at the indicated incubation time and processed for SDS-PAGE and immunoblotting. Blots were probed successively with anti-cleaved-caspase 7, -caspase 3, -PARP, and Erk1/2 antibodies. (b) Densitometric and statistical analyses of the results shown in a on day 7. Data show fold-changes for each protein in treated samples as compared with the corresponding NT control. ODN, oligodeoxynucleotide.

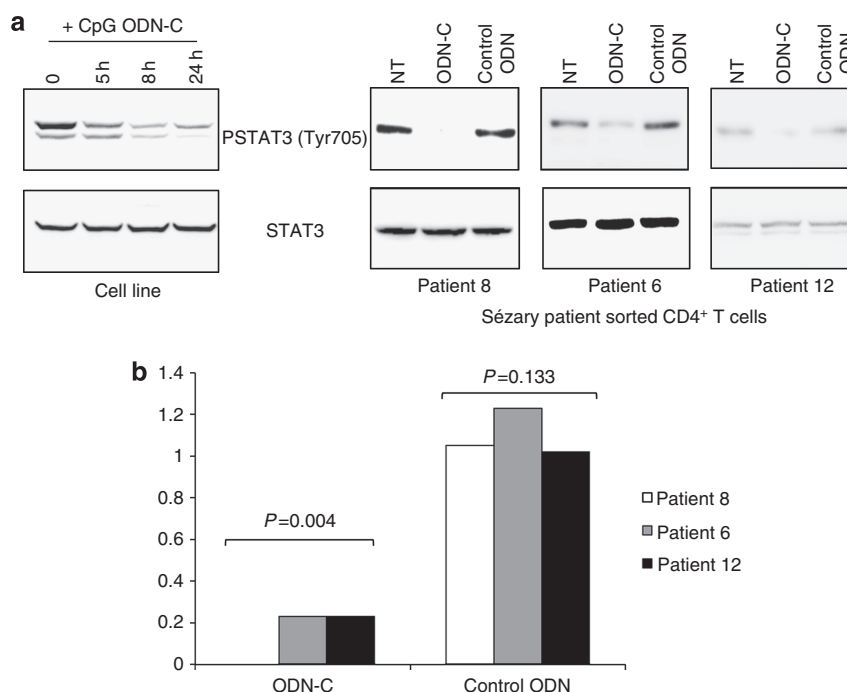


Figure 5. Dephosphorylation of STAT3 upon ODN-C treatment of Sézary cells. (a) The Sézary cell line Pno or sorted CD4⁺ T cells from Sézary patients were treated as in Figure 4. Blots were then revealed using anti-phospho-STAT3 antibodies, dehybridized, and reprobed with STAT3 antibodies. (b) Densitometric and statistical analyses of the immunoblots shown in a. ODN, oligodeoxynucleotide; STAT3, signal transducer and activator of transcription 3.

Sézary cells (activating receptor vs. apoptosis-mediating receptor, respectively), depending on TLR9 expression.

An alternative to overcome the lack of TLR9 in Sézary cells could be the involvement of cytosolic DNA receptors such as the cytosolic GMP-AMP synthase or absent in melanoma-2 (AIM2) following KIR3DL2/ODN-C internalization. However, an interaction between cytosolic GMP-AMP synthase and CpG ODN-C seems unlikely as cytosolic GMP-AMP synthase showed no or low affinity for double-stranded DNA ligand shorter than 15 base pairs or encompassing a phosphorothioate backbone (Karayel *et al.*, 2009; Kranzusch *et al.*, 2013), both features that are shared by ODN-C. In contrast, AIM2 is a member of the IFN-inducible HIN-200 family, whose DNA-binding specificity allows double-stranded DNA with a phosphorothioate backbone as ligand (Burckstummer *et al.*, 2009). It has been shown that the interaction of AIM2 with double-stranded DNA led to its association with the apoptosis-associated speck-like protein containing a CARD, resulting in caspase-1 activation and pyroptotic cell death (Fernandes-Alnemri *et al.*, 2009). Although our results rather suggest that KIR3DL2/CpG ODN-mediated cell death occurs through a classical apoptotic pathway involving caspases 3 and 7, the possibility of AIM2 expression upon ODN-C treatment of Sézary cells was investigated. Experiments performed on the KIR3DL2⁺ Sézary cell line HUT78 showed no induction of AIM2 expression upon ODN-C treatment despite effective cell apoptosis (data not shown), excluding the participation of AIM2 in the KIR3DL2/CpG ODN-dependent pathway.

Several studies aimed to induce Sézary cell apoptosis mainly through the use of drugs directed against targeted proteins (e.g., HDAC, Janus kinase (JAK) or γ -secretase inhibitors) (Eriksen *et al.*, 2001; van Kester *et al.*, 2008; van der Fits *et al.*, 2012). Such inhibitors have proved their efficacy in inducing Sézary cell apoptosis *in vitro*, with the detection of 20–100% of dead cells after 48 hours of treatment at the highest concentration tested, with a usually better response for cell lines than for patients' circulating cells. Experiments performed on the proliferating Sézary cell line Pno (and HUT78; data not shown) evidenced an internalization of the receptor 6–8 hours after addition of the CpG ODN with STAT3 dephosphorylation and cell apoptosis readily detected after 24 hours. By comparison, the delay observed for detecting an ODN-C/KIR3DL2-mediated apoptosis of the patients' tumoral clone seems unusual. Indeed, despite maximal but partial internalization of KIR3DL2 at 24–48 hours after exposure to ODN-C, and STAT3 complete dephosphorylation after 3 days of treatment, detection of the patients' circulating tumoral T cell clone apoptosis required 7–12 days of treatment. Further studies will be needed to determine whether these time-course discrepancies might be the consequence of the low metabolic turnover of peripheral blood tumoral cells compared with an established and proliferating Sézary cell line, and the need for days to shut down phospho-STAT3-dependent anti-apoptotic pathways. It has been reported that the constitutive activation of STAT3 in cutaneous T cell lymphoma cell lines led to a constitutive expression of SOCS3 (Brender *et al.*, 2001). However, we did not observe a diminished or abolished expression of SOCS3 upon ODN-C

treatment of Sézary cells at time points at which STAT3 inactivation was readily detected (data not shown). One can therefore wonder whether an above normal protein stability could be a hallmark of patients' malignant T cells. Efforts will be made to further determine the consequences of the ODN-C/KIR3DL2 internalization in terms of intracellular molecular events to understand the precise time line that leads to delayed STAT3 dephosphorylation and Sézary cell death. In this regard, previous studies demonstrated that treatment of Sézary cells with the JAK inhibitor tyrphostin AG490, as well as with Cucurbitacin I or Curcumin, efficiently promotes phospho-STAT3 dephosphorylation and induces Sézary cell apoptosis (Eriksen *et al.*, 2001; Krejsgaard *et al.*, 2006; van Kester *et al.*, 2008; Zhang *et al.*, 2010). It has been recently established that constitutive activation of STAT3 in Sézary cells is not due to a loss of SHP-1 but is mediated by a constitutive aberrant activation of JAK family members (McKenzie *et al.*, 2011). Work is in progress to determine whether CpG ODN binding to KIR3DL2 in Sézary cells may influence JAK activity.

Phase I/II trials using TLR9 agonists alone or combined with localized low-dose radiotherapy have been conducted on MF and SS patients (Kim *et al.*, 2010, 2012). In both studies, class-B CpG ODN was injected subcutaneously and led to a clinical response rate of 32–36% with no major cytotoxic side effects. Moreover, whereas CpG ODN induced lesion clearing, significantly decreased CD25⁺ FoxP3⁺ regulatory T cells and S100⁺ antigen-presenting cells, and increased levels of CD123⁺ plasmacytoid dendritic cells at the sites of injection, its effects on the circulating tumoral burden of SS patients were not explored. Our data suggest that, as well as promoting the generation of an anti-tumoral immune response, CpG ODN might also initiate a direct effect on Sézary cells by binding to KIR3DL2.

MATERIALS AND METHODS

Patients and cells

SS diagnosis was established on recognized international clinical, histological, and biological criteria. After obtaining written informed patient consent, blood from 15 SS patients with more than 90% of CD3⁺CD4⁺KIR3DL2⁺ cells was collected for this study, which was approved by the institutional ethics committee (Saint Louis Hospital, Paris). PBMCs were isolated from heparinized venous blood by density gradient centrifugation over lymphocytes separating medium (LSM; PAA Laboratories, Les Mureaux, France). CD4⁺ T cells were purified from freshly isolated PBMCs by magnetic activated cell sorting using the CD4⁺ T cell isolation kit according to the manufacturer's protocol (Miltenyi Biotech, Paris, France) and immediately subjected to CpG ODN treatment (see below). The IL2-dependent Sézary cell line (Pno) used in this study was established and amplified as described previously, and maintained its phenotype (Poszepczynska *et al.*, 2000). Cells were cultured in RPMI-1640 supplemented with 2 mM L-glutamine, 1% penicillin-streptomycin (Invitrogen, Saint Aubin, France) and 10% human serum (Jacques Boy Biotechnologies Institute).

CpG ODN cell treatment

Cells were cultured for the indicated time in 24-well plates at a concentration of 2×10^6 per ml. The following CpG ODNs were used

at a final concentration of $10\mu\text{g ml}^{-1}$: class-A (ODN 2336), class-B (ODN 2006), class-C (ODN 2395), and control ODN (ODN TTAGGG) (all from Invivogen, Toulouse, France). Cells were incubated for up to 12 days. After incubation, cells were processed for flow cytometry or biochemical analysis, as described below. Where indicated, cell viability was assessed by Trypan blue exclusion.

Flow cytometry

Sézary cell staining was performed according to a standard procedure using the anti-KIR3DL2 mAb Q66 (IgM; kindly provided by Dr A Moretta, Genova, Italy) plus goat anti-mouse IgM-FITC (or -PE) Abs, anti-CD3-PC7, -TCRV β -FITC (or -PE), and -CD4-PC5 mAbs (Beckman Coulter, Marseille, France). Detection of apoptotic cells was performed using 7AAD (BD Biosciences, Le Pont de Claix, France), according to the supplier's protocol. Cells were analyzed on an FC500 cytometer (Beckman Coulter) and data analyzed with the FlowJo software.

Immunofluorescence

Cells were either left untreated or incubated in the presence of CpG ODN-C, FITC-labeled CpG ODN-C, or control ODN at 37 °C. Cells were then washed and immobilized on poly-L-lysine-coated coverslips. After a methanol fixation step at -20°C and permeabilization in phosphate-buffered saline/0.1% Tween-20, anti-KIR3DL2 (Q66, mouse IgM), -LAMP-1 (rabbit IgG, Millipore, Molsheim, France), or/and FITC-ODN-C were added and revealed with the appropriate Alexa488- or Texas Red-coupled secondary antibodies. After washes, coverslips were mounted in DAPI Fluoromount-G (Southern Biotech/Clinisciences, Nanterre, France) and analyzed on a Leica DMRB microscope (Le Pecq, France).

Immunoprecipitation and western blotting

Untreated or ODN-treated cells were lysed, and post-nuclear supernatants were prepared and processed as described elsewhere (Bensussan *et al.*, 2011). For western blotting, samples were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. Analyses were performed using antibodies specific for the following molecules: cleaved-caspase 3, -caspase 7, and -PARP, phospho-STAT3, STAT3, and Erk1/2 (all from Cell Signaling Technology/Ozyme, Saint Quentin en Yvelines, France). When performing detection in series, a stripping procedure between each revelation step was carried out. After incubation with the appropriate horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch/Beckman Coulter), detection was performed using an ECL system (Perbio Science, Brébières, France) and an ImageQuant LAS 400 system (GE Healthcare, Vélizy-Villacoublay, France).

Statistical analysis

Statistical analyses were performed using Student's *t* test (one tailed, paired). The given *P*-values for each condition correspond to the comparison with untreated controls. For immunoblots, protein band intensity was quantified using the Image Quant TL software (GE Healthcare). Data were then expressed as fold-changes in treated samples as compared with non-treated controls.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/jid>

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